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(71) Applicant: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West Seventh Street, Austin, TX 78701 (US).

(72) Inventors: PATHAK, Sen ; 927 Summers Drive, Missouri City, TX 77489 (US). ARDEN, Karen, C. ; 4390 Grand Blvd. ,110, Montréal, Québec H4B 2X8 (CA).

(74) Agent: PATTERSON, Melinda, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

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(54) Title: METHOD FOR DIAGNOSIS AND MONITORING OF TUMORS

(57) Abstract

Methods for diagnosing and monitoring tumor cells employ analysis of silver staining in interphase nuclei. In a preferred embodiment, the invention concerns the ratio of silver-stained area to total nuclear area of interphase cells, quantitatively determined by computer-assisted microscopy.

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METHOD FOR DIAGNOSIS AND MONITORING OF TUMORS

This invention relates to methods for diagnosing and monitoring tumors and employs analysis of silver staining in interphase nuclei. In a preferred embodiment, the invention concerns the ratio of silver-stained area to total nuclear area of interphase cells, quantitatively determined by computer-assisted microscopy.

For some years, cytogeneticists have used an argentophilic silver staining technique to examine Nucleolus Organizer Regions (NOR) (DNA regions containing cistrons for ribosomal RNA) in metaphase chromosome spreads. This technique has been shown to stain proteins that apparently become associated with NORs during the interphase period of the cell cycle. In humans, the NORs are situated in the short arms of chromosomes 13, 14, 15, 21 and 22; consequently, there are 10 NOR-bearing chromosomes in a normal diploid methaphase spread. It is rare, however, to find a metaphase spread in which all NORs are stained.

In past studies, the present inventors demonstrated that the number of silver-stained NORs in metaphase bone marrow cells obtained from leukemic patients differs substantially from the number observed in similar cells from normal patients. (Arden, et al., 1985) Moreover, the number of NORs stained per metaphase and the percentage of metaphase cells that were stained was found to vary with the stage of disease. Therefore, it was believed that silver staining of metaphase cells would prove useful in diagnosing or monitoring cancerous diseases, particularly malignancy of the reticuloendothelial system.

Unfortunately, analysis of metaphase staining possessed disadvantages that made it less than ideally suited for widespread clinical application. For example, with certain patients, it was difficult to obtain a sufficient number of metaphase cells to conduct a meaningful analysis. Although interphase cells exhibiting a degree of silver staining were generally abundant in samples from those patients, the clinical significance of such staining was unclear.

Attempts to clarify the significance of interphase staining produced conflicting reports. For example, some studies indicated that a decrease in the intensity of silver staining of the nucleoli in interphase cells correlates with inhibition of rRNA synthesis but the number of silver staining NORs observed in metaphase cells does not (Hofgartner, et al., 1979). In contrast, other studies indicated that interphase nucleolar activity correlates with the number of silver staining NORs in metaphase cells. (Lau and Arrighi 1976; Lau, et al., 1978).

Moreover, techniques used by others to quantitate staining, including counting individual silver grains or

visually "ranking" the amount of silver-stained per interphase nucleus, were tedious, subjective and of questionable accuracy when applied to interphase cells. Therefore, considerable room for improvement remained.

Recognizing these other short comings of the prior art, the present inventors sought to develop a method by which the silver staining technique could be used to reliably assay cells in interphase from individuals suspected of having tumors.

Therefore, in accordance with the present invention, there are now provided several novel methods for assaying a population of cells from a patient suspected of having a cancerous disease. The first of these general methods entails obtaining a sample of the cells to be tested; depositing them on a slide; staining said cells with a staining preparation including silver; identifying a stained interphase cell in said sample; and measuring the relative areas of the nucleus of the cell and the silver stained portion of the nucleus to obtain a ratio of silver stained area to nuclear area. If desired, the identifying and measuring steps may be repeated for a selected number of times, for example, ten or more times, in order to ensure statistical accuracy. It is usually preferable that the procedures be performed on at least about 25-50 cells. The number of ratios thus obtained may then be averaged to provide an average ratio of silver stained area to nuclear area for the cell population assayed. If desired, this ratio may then be compared to a standard ratio, or control value, in order to determine whether or not the ratio obtained for the patient's cells is significantly different from that obtained from cells from a comparable normal tissue, for example, from a normal individual.

In other aspects, the invention provides a second assay method that has the advantage of being easier to perform than the method described above; however, in some circumstances, it may be somewhat less accurate since it does not correct for heterogeneity of nuclear size. This method, also directed to assaying cells from an individual suspected or diagnosed as having cancer, comprises obtaining a sample of cells to be assayed; depositing them on a slide; staining said cells with a staining preparation containing silver; identifying a stained interphase cell in said sample; and measuring the relative area of the silver-stained portion of said cell. As indicated previously, the identifying and measuring steps may be repeated a number of times and the areas obtained averaged to produce a mean silver-stained area for the cell population. If desired, the mean silver-stained area may be compared to a control or standard value. Such standard value or control may be obtained, for example, by analyzing similar cells from homologous normal tissue, for example, from a normal individual. Those of skill in the art are familiar with the concept of controls or standard values and will be able to devise appropriate methods for obtaining appropriate standard values without the need for undue experimentation.

As those skilled in the art will also recognize, the invention is not dependent on obtaining actual area measurements of silver-stained portions of the cell. Therefore, the term area is not to be narrowly construed; relative area measurements, for example (those that can be compared one to another or to a reference value) are quite acceptable for purposes of the invention.

In general, it is predicted that the method of the present invention is likely to prove most useful in diagnosing or monitoring cancerous diseases, for example,

the tumor cells of the reticuloendothelial system. As known by those in the art, certain of these cancerous diseases are generally likely to result in leukemia or lymphoma. Thus, in a preferred embodiment, the cells to be assayed are cells of the reticuloendothelial system, such as, for example, blood cells, bone marrow cells, spleen cells, lymph node cells, etc. In an even more preferred embodiment, the cells to be assayed are bone marrow cells and the disease is leukemia.

Generally, the cells to be assayed may be taken from the individual by biopsy, phlebotomy, or other known techniques; and dispersed or deaggregated, if necessary. These cells may then be placed on a microscope slide. The sample may be deposited on the slide by any of a number of methods known to those skilled in the art. Such techniques include, but are not limited to, centrifugation onto a microscope slide, for example, by cytocentrifuge; preparation of a "standard" hematological smear by spreading a suspension of cells out on the surface of a microscope slide with the edge of a second slide; placing a drop of a cell suspension to be tested on an area of a slide and allowing it to air dry; and any of a number of other variations generally known to those of skill in the art, including preparation of tissue sections, for example, with a microtome. Moreover, for purposes of the present invention, the term slide is to be broadly construed as any surface on which cells may be deposited and examined by microscope. Such surfaces include, but are not limited to, standard microscope slides, cover slips, tissue culture dishes and plates or even simple pieces of transparent glass or plastic.

Finally, it should be appreciated that the "depositing" and "staining" steps do not have to be performed in any particular order. In certain instances

one may wish to stain a suspension of the cells first, then deposit the stained cells on the slide. However, the preferred procedure is to deposit cells first on slides and then staining them with silver nitrate solution.

The slide is then examined by microscope and an interphase cell identified. Of course, to those of ordinary skill in the art, identification of a given cell as an interphase cell should usually be rather straightforward. The silver-stained area of the interphase cell is then measured. Several methods or devices may be used for measurement so long as they allow one to accurately and reproducibly measure the area of silver staining. The present inventors have discovered that acceptable area measurements can be made using a microscope equipped with a computer (i.e.--computer-assisted microscopy) suitable for measuring relative areas of cells and the like. Therefore computer-assisted microscopy is preferred as a method for measuring area.

Figure 1. Silver staining in bone marrow interphase nuclei from a) normal subject and b) acute lymphocytic leukemia (ALL) patient.

Figure 2. Scatter diagrams showing the correlation between the mean silver-stained area per nucleus and a) the percentage of silver-stained metaphases and b) the mean number of Ag-NORs per metaphase.

Figure 3. Scatter diagrams showing the correlation between the mean percentage of silver-stained area per nucleus and a) the percentage of silver-stained metaphases and b) the mean number of Ag-NORs per metaphase.

The present invention employs the inventors' discovery that by quantitating the area of silver staining

in interphase cells, one can facilitate detection and monitoring of tumors.

In developing the invention, the present inventors have used bone marrow cells from patients with acute lymphocytic leukemia (ALL) as a model system. However, it is likely that the techniques of the invention will be equally useful in diagnosis and monitoring of other tumors or cancers such as those of the skin, gonads, or other solid tumors including reticuloendothelial system. Such cancers may include, for example, chronic lymphocytic leukemia (CLL), acute myologenous leukemia (AML), chronic granulocytic leukemia (CGL), Non Hodgkin's lymphoma, Burkitt's lymphoma other types of hematologic malignancies, gonadoblastoma, and melanoma. Tissues for which the method is likely to prove particularly useful include bone marrow, peripheral blood, spleen, lymph node, skin fibroblasts, and gonadal cells.

In general, it will often be advisable to select the tissue that will serve as the source of the cells to be assayed in view of the particular disease that the patient is suspected of, or diagnosed as, having. For example, with ALL, the preferable tissue source will be bone marrow; with CLL, blood or spleen may be preferred. Similarly, with melanoma, skin fibroblasts are preferred for examination and with gonadoblastoma, gonadal cells. Of course, with the aid of hindsight afforded by the present disclosure, those of skill in the art may discover other suitable combinations that should be considered to be within the scope of the present invention.

The cells to be assayed may be stained by the basic silver staining technique developed by Goodpasture and Bloom (1975) or any of a number of suitable modifications thereof. For example, a suitable modified staining

procedure is described by Pathak and Elder (1980). However, a preferred procedure is set forth in detail in the Example presented below.

Similarly, it may be possible to measure the area of silver staining in several ways. However, computer-assisted microscopy like that described in the Example represents a highly preferred method. Of course, virtually any computer-assisted microscope capable of measuring relative areas may be used.

The following Examples are provided in order to demonstrate practice of certain aspects of the present invention. Accordingly, they should not be construed as limiting the scope of the claims unless distinctly specified therein.

Example 1

Analysis of Silver-Stained Bone Marrow Smears From Patients Having Acute Lymphocytic Leukemia (ALL) and Normal Individuals

A. Preparation and Staining Of Bone Marrow Smears

Bone marrow samples were obtained from six untreated ALL patients, three normal individuals, and one bone marrow transplant recipient in culture medium (RPMI-1640). The samples were incubated for 24 hours at 37° in RPMI-1640 supplemented with 15% fetal bovine serum. Prior to harvesting, Colcemid (final concentration 0.4 µg/ml) was added for 30 minutes. After centrifugation the cells were incubated in 0.06 M KCl for 20 minutes at 37°C, pelleted, and fixed three times in a methanol/glacial acetic acid mixture (3:1 by volume). The cells were then deposited on

slides, allowed to air dry, and aged for at least twenty-four hours.

For silver staining, the slides were first rinsed in borate buffer (pH 9.1) for 20 minutes, then rinsed in deionized water and then flooded with 50% aqueous silver nitrate solution containing 0.03% formalin. Slides were incubated in a moist chamber either for 16-20 hours at 55°C or for 2 hours at 65°C. When the desired intensity of silver staining had been achieved, the slides were brought to room temperature, rinsed in deionized water, and counter stained for a few second in 1% Giemsa. The slides were rinsed with deionized water and allowed to air dry (Pathak and Elder, 1980).

The stained cells were then analyzed as follows. First, individual metaphase cells in each sample were analyzed for the presence or absence of silver staining as well as the number of silver-stained NORs per cell. The percentage of metaphases stained with silver and the average number of Ag NORs per metaphase was recorded for each sample.

Interphase cells were examined by a protocol utilizing computerized photomicroscopy to quantitate relative areas of silver staining to total nuclear area. In the instant case, the procedure was performed with a Ziess 1 BAS image analysis system (Carl Ziess, Inc., Thornwood, New York). This system comprises of a Zeiss Universal photomicroscope, a Dage-MTI Series 66 camera, Kontron computer with image array processor and a Zeiss software package for performing image enhancement and measurement of relative areas. The slides were scanned with the photomicroscope using a 100X oil objective. Fifty interphase cells from random areas of the slide were examined. The raw image of the cells was electronically

enhanced to increase contrast and magnified 2.5 times before measurements were made. The following parameters were measured: (a) the area occupied by the nucleus, (b) area occupied by the silver stain, and (c) the percent area occupied by the silver stain relative to the nucleus. Area was measured in μm^2. For all analyses, the slides were coded before data collection and decoded at the completion of the study.

More specifically the steps involved in the particular computer-assisted image analysis measurement performed by the present inventors were as follows: First, a photomicrograph of a selected interphase cell was displayed on a video screen. The computer program was then used to enlarge the image of the cell to 2.5 times its original size, and the silver-stained areas of the nucleus were shaded in with a continuous grey scale. Non-shaded areas were then electronically removed from the screen, the shaded area to be measured was circled, and the remainder of the shaded areas were subtracted from the image. The areas remaining were measured. Then, the enlarged, enhanced image was returned to the screen and the entire nucleus shaded. Non-shaded areas were again subtracted from the image and the nucleus filled. The area to be measured was indicated by removing the color and the total area of the nucleus was then calculated. The procedure was then repeated for a number of other interphase cells.

B. Statistical Analysis

The data were analyzed on scatter plots, and a regression coefficient was calculated for each distinct diagnostic group. In addition, the Wilcoxon-Mann-Whitney test (Zar, 1984) was performed to detect differences in the number of NORs per metaphase between the control group

and the ALL group. In order to study the percentages of silver-stained metaphases, we transformed each percentage by the arc-sine method. A t-test was then performed using the transformed data to examine the differences between the two diagnostic groups. A one-way analysis of variance with the Student-Newman-Keuls multiple range test was employed to compare the measurements of silver-stained areas obtained by image analysis with the metaphase data.

C. Results of Silver Staining of Normal and Leukemic Cells

Silver-stained NORs in metaphase cells from ALL patients are discrete and very easily located and counted. Silver-stained interphase nuclei from normal and leukemic bone marrow cells are shown in Figure 1. The interphase nuclei from normal individuals show fewer and smaller silver-stained areas than those from ALL patients; interphase nuclei from the latter show an increase in the silver-stained areas as well as larger "spots" of silver staining.

Table 1 provides a comparison of stained metaphase NORs to the area of staining in interphase nuclei. In both normal individuals and ALL patients, a proportion of metaphases were not stained with silver despite the presence of silver staining in the neighboring interphase nuclei. The fraction of positively stained metaphases was considerably higher among the ALL patients when compared to the normal controls. The percentage of Ag-NOR positive metaphases in the ALL patients ranged from 63.3% to 96.0%; while that of the control group ranged from 22.0% to 44.0%. In addition, after arc-sine transformation, the average percentage of Ag-positive metaphases for the control group and the ALL group were significantly different ($P < 0.0001$). The average number of silver-

stained NORs per metaphase was also consistently lower in the control group than in the ALL group. The mean number of Ag-NORs in the leukemic group was statistically different from that of the control group ($P < 0.0001$ by Wilcoxon-Mann-Whitney test).

The results obtained with interphase nuclei parallel the results obtained with metaphase cells. The average silver-stained area per nucleus was consistently higher in the leukemic patients than in the control individuals. When the silver-stained area was expressed as a fraction of the total nucleus area, significant difference between the control group and the ALL group was also observed. Using a Oneway analysis of Variance, with each patient as a separate group, it was determined that the means were different for the control group and the leukemic group ($P < 0.0001$).

The mean silver-stained area per nucleus and the fraction of the nucleus stained with silver were also tested to determine the existence of significant differences between samples of cells from individual patients. By the Student-Newman-Keuls test, no differences between the four control samples were found; the ALL interphase nuclei were more heterogenous and did differ (see the last column in Table 1.) However, each ALL sample was significantly different from the control samples. ($P. > 0.05$) This was true for both the mean silver-stained area per nucleus and the percentage of the nucleus stained with silver.

In an additional analysis, subsets of groups whose highest and lowest means did not differ by more than the shortest significant range for a subset of that size were determined. Using this criterion, the four control samples again differ from the ALL samples. This was true

for both the mean silver-stained area and the fraction of the nucleus stained with silver.

The positive correlation between the fraction of Ag-positive metaphases and the mean silver-stained area in interphase nuclei is shown in Figure 2a (correlation coefficient 0.83). Mean number of Ag-NORs per metaphase also correlated with silver-stained area as shown in Figure 2b (correlation coefficient 0.82). Despite a number of individual variations, it is clear that the control samples are separated from the samples obtained from ALL patients.

When the mean percent silver-stained area was measured, the correlation with the fraction of Ag-positive metaphases was even better (Figure 3a, correlation coefficient of 0.97). Similarly, the correlation between the mean number of Ag-NORs per metaphase and the mean percent silver-stained area, with a correlation coefficient of 0.97, was higher than that of the number of Ag-NORs per metaphase and the mean silver-stained area (Figure 3b). Therefore, these four comparisons demonstrate a strong correlation between the metaphase and interphase data on silver staining.

Table 1: Results of silver staining in bone marrow metaphases and interphase nuclei. (a,b.c,d = the four significantly different groups)

Samples	Status	% Ag+ metaphases	Average No. NOR per metaphase	Average silver area per nucleus	Average % silver area per nucleus	Results of stat. test
BL-1422a	normal	22.0	0.54 (1.26)	5.69 (6.47)	1.32 (0.66)	a
BL-2164a	normal	41.2	1.00 (1.28)	5.72 (6.05)	1.80 (1.44)	a
BL-2805a	normal	36.0	1.72 (2.62)	9.58 (8.08)	1.92 (1.22)	a
BL-2885a	normal	44.0	1.19 (2.45)	8.40 (9.38)	1.55 (1.29)	a
BL-2381a	ALL	78.0	1.82 (1.47)	22.67 (12.34)	3.60 (1.16)	b
BL-2197a	ALL	63.3	2.59 (2.35)	22.25 (13.22)	4.12 (2.19)	b,c
BL-2213a	ALL	80.0	4.00 (2.23)	16.42 (11.07)	4.20 (2.89)	b,c
BL-2681a	ALL	68.0	4.04 (3.31)	31.12 (16.11)	4.06 (1.80)	b,c
BL-1980a	ALL	96.0	3.02 (1.18)	21.30 (10.22)	4.91 (2.16)	c
BL-2044a	ALL	92.0	4.50 (1.79)	14.45 (8.99)	6.16 (3.70)	d

N + normal bone marrows; ALL = acute lymphocytic leukemia marrows.

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UTSC:088/C4

CLAIMS:

1. A method for assaying cells from an individual suspected of having a cancerous disease, comprising:

a) obtaining a sample of the cells to be assayed;

b) depositing said sample on a slide;

c) staining said cells with a staining preparation containing silver;

d) identifying a stained interphase cell in said sample;

e) measuring the relative areas of the nucleus of said cell and the silver-stained portion of said nucleus to obtain a ratio of silver-stained area to nuclear area;

f) repeating steps (d) and (e); and

g) averaging the ratios obtained to provide an average ratio of silver-stained area to nuclear area.

2. The method of claim 1, wherein:

a) the cells to be assayed are cells of the reticuloendothelial system; and

b) said cancerous disease is leukemia or lymphoma.

3. The method of claim 1 wherein said cells are bone marrow cells and said cancerous disease is leukemia.

4. The method of claim 1 wherein steps (d) and (e) are repeated at least about 25 times.

5. The method of claim 1 wherein the steps (d) and (e) are performed by computer-assisted microscopy.

6. The method of claim 1 wherein the silver used to stain the cells is silver nitrate.

7. A method for assaying cells from an individual suspected having cancer, comprising:

(a) obtaining a sample of the cells to be assayed;

(b) depositing said sample on a slide;

(c) staining said cells with a staining preparation containing silver;

(d) identifying a stained interphase cell in said sample;

(e) measuring the area of the silver-stained portion of said interphase cell;

(f) repeating steps (d) and (e) on selected number of cells; and

(g) calculating a mean silver-stained area.

8. The method of claim 7, wherein:

a) said cells are cells of the reticuloendothelial system; and

b) said cancerous disease is leukemia or lymphoma.

9. The method of claim 7 wherein said cells are bone marrow cells and said disease is leukemia.

10. The method of claim 7 wherein steps (d) and (e) are repeated at least about 25 times.

11. The method of claim 7 wherein the steps (d) and (e) are performed by computer-assisted microscopy.

12. The method of claim 7 wherein the silver used to stain the cells is silver nitrate.

FIG.1a

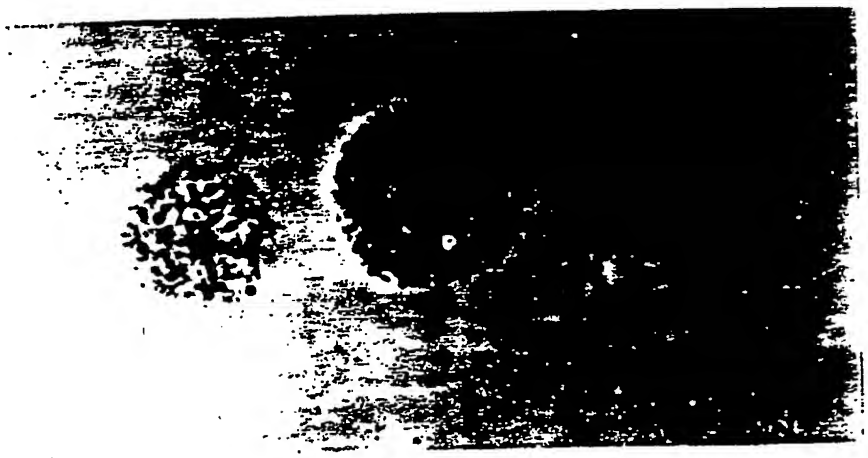


FIG.1b

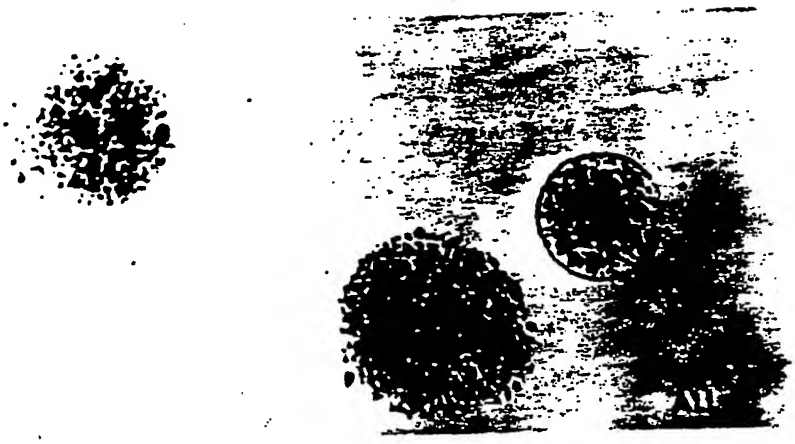
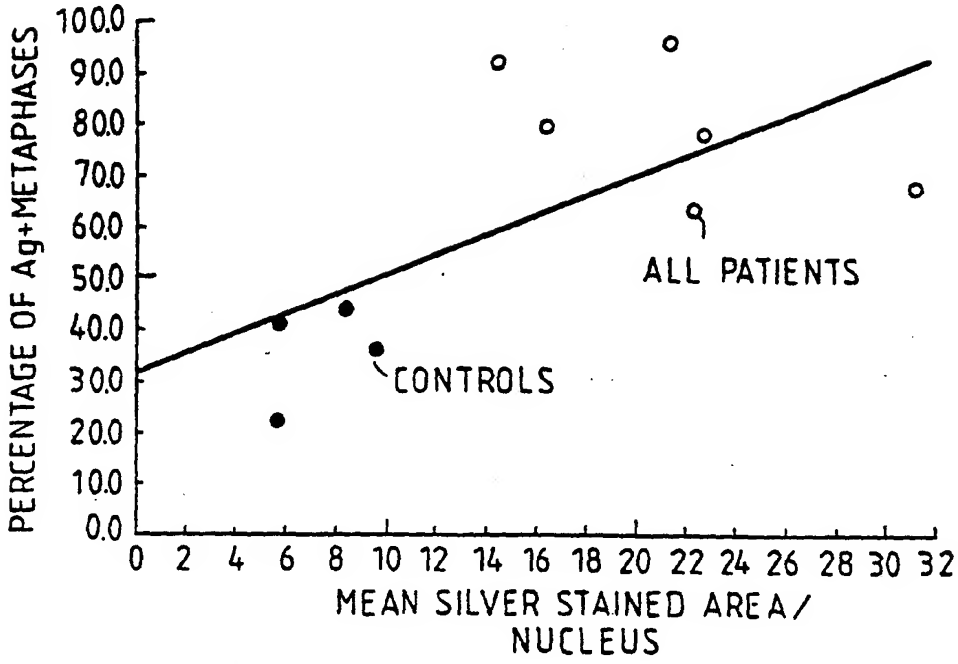


FIG.2a


PERCENTAGE OF Ag+METAPHASES
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90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
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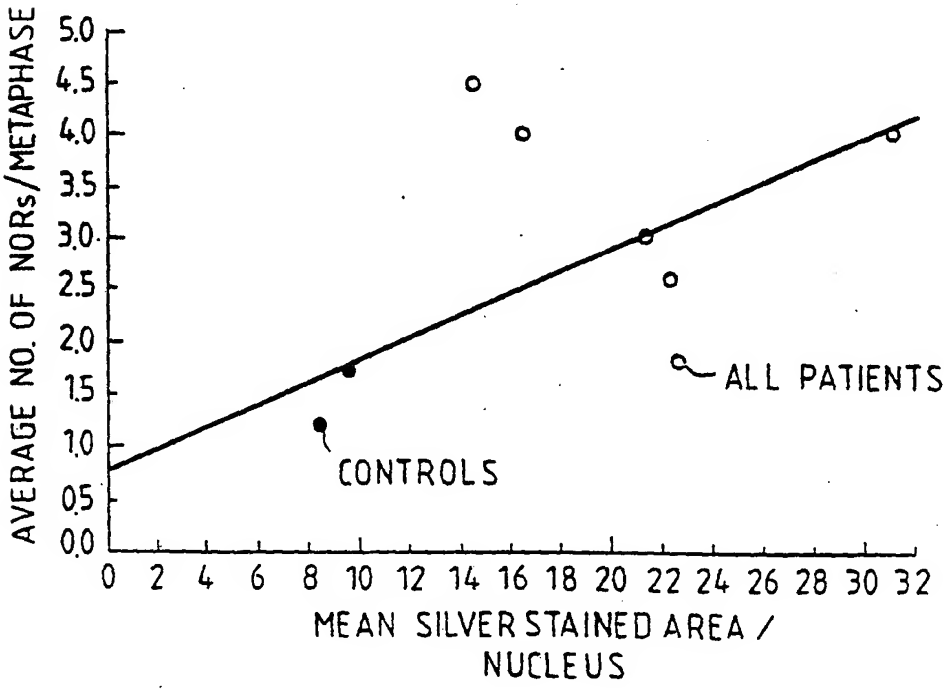
MEAN SILVER STAINED AREA/ NUCLEUS
ALL PATIENTS
CONTROLS

FIG.2b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4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
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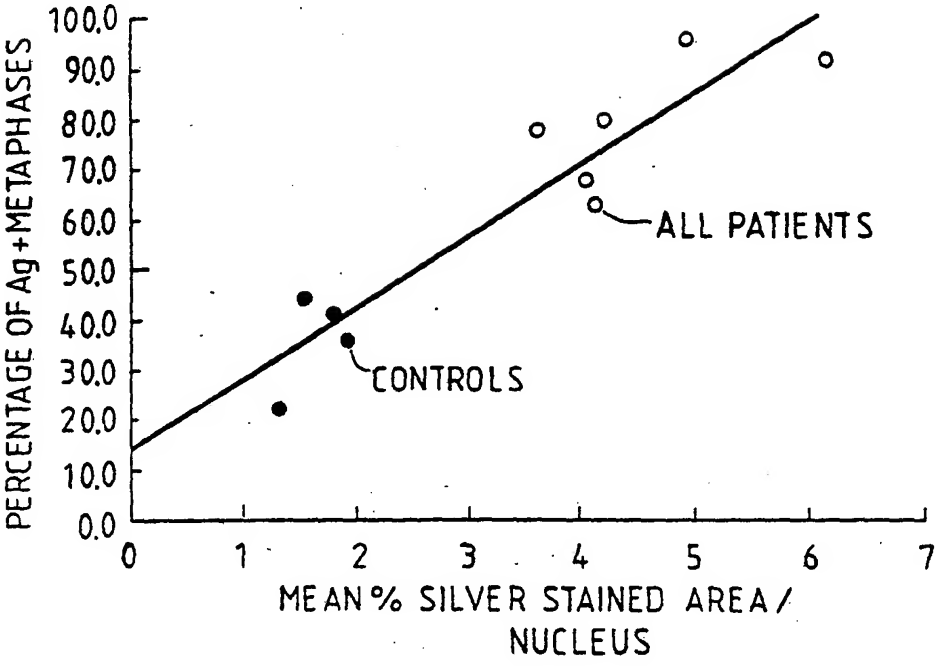
MEAN SILVER STAINED AREA / NUCLEUS
ALL PATIENTS
CONTROLS

FIG. 3a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60.0
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30.0
20.0
10.0
0.0
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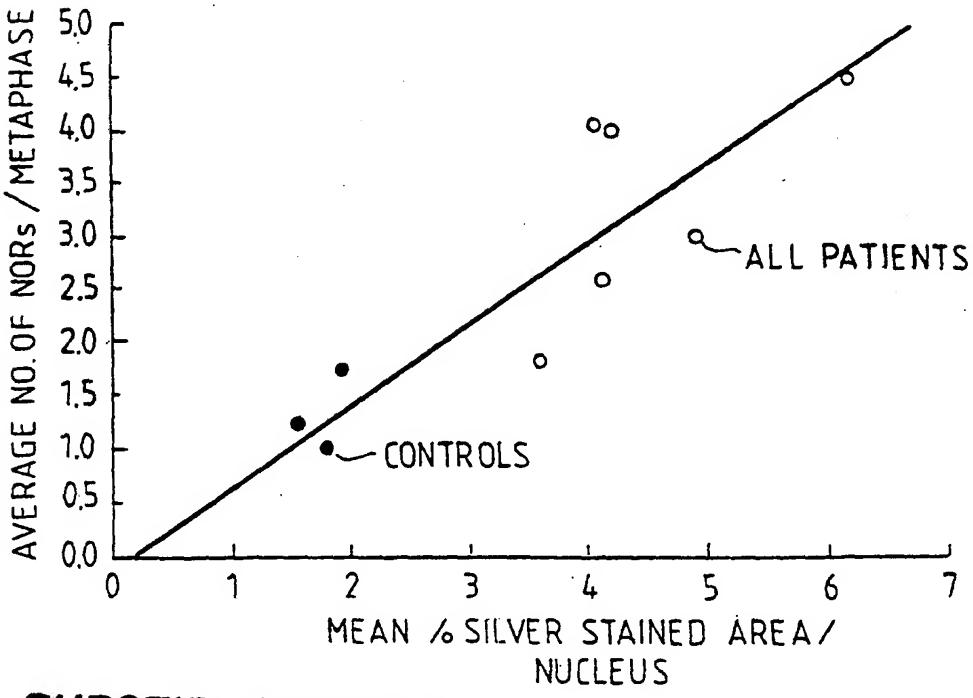
1
2
3
4
5
6
7
ALL PATIENTS
CONTROLS
MEAN % SILVER STAINED AREA / NUCLEUS

FIG. 3b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0
1
2
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ALL PATIENTS
CONTROLS
MEAN % SILVER STAINED AREA / NUCLEUS

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03500

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: G 01 N 33/574, G 01 N 1/30

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Biological Abstracts, volume 78, no. 8, 1984, (Philadelphia, PA, US), L. Manuelidis: "Active nucleolus organizers are precisely positioned in adult central nervous system cells but not in neuroectodermal tumor cells", see page 6931, abstract 61495, & J. Neuropathol. Exp. Neurol. 43(3): 225-241, 1984 --	1-12
Y	Clinical Chemistry, volume 28, no. 4, April 1982, (Winston, Salem, US), S.M. Hanash et al.: "Two-dimensional gel electrophoresis of cell proteins in childhood leukemia, with silver staining: a preliminary report", pages 1026-1030 see pages 1026-1030 --	1-12
Y	Biological Abstracts, volume 67, no. 7, 1979, (Philadelphia, PA, US), R.T. Tateishi et al.: "Demonstration of argyrophil granules in small cell	1-12

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th December 1989	05 FEB 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	C.D. v.d. Vliet

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	carcinoma of the lung", see pages 4222-4223, abstract 42515, & Virchows Arch. A. Pathol. Anat. Histol., 377 (3): 203-210, 1978 --	
Y	Chemical Abstracts, volume 99, no. 22, 28 November 1983, (Columbus, Ohio, US), E. Rengifo et al.: "Ammoniacal silver staining of lymph node cells. I. Its potential value for the diagnosis of Hodgkin's disease", see pages 406-407, abstract 190912p, & Neoplasma 1983, 30(4), 469-73 --	1-12
Y	Chemical Abstracts, volume 90, no. 22, 28 May 1979, (Columbus, Ohio, US), L.-M. Shi et al.: "Cytochemical observations on the chromosomal nucleolar organizer region in mouse ascites tumor cells using silver staining", see page 460, abstract 184508j, & K'o Hsueh T'ung Pao 1979, 24(2), 93-6 --	1-12
Y	Chemical Abstracts, volume 91, no. 18, 20 October 1979, (Columbus, Ohio, US), M.A. Lischwe et al.: "Proteins C23 and B23 are the major nucleolar silver staining proteins", see page 387, abstract 154865b, & Life Sci. 1979, 25(8), 701-8 --------	1-12